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14. ABSTRACT We proposed to investigate the role of Bmi-1 (a member of polycomb gene family) in human prostate cancer (CaP) development. Here, we present the work accomplished during the first year of the project. Immunohistochemical analysis of prostatic specimens of 125 human CaP patients showed that Bmi-1 protein levels are highly elevated in patients with advanced disease. To understand the mechanism of action of Bmi-1, we employed two-prong strategy. Firstly, Bmi-1 was knocked down in CaP cells (LNCaP, DU145 and PC-3) by employing siRNA technique. Bmi-1-silenced CaP cells exhibited decreased proliferative and clonogenic potential. Secondly, Bmi-1 was over-expressed in CaP cells by transfecting Bmi-1 overexpressing plasmid (pbabe-Bmi-1) in CaP cells. Bmi-1-overexpressing cells exhibited increased clonogenicity and rate of proliferation. Based on the outcome of micro-array analysis, we analyzed CaP cells for Cyclin D1 (the cell cycle regulatory protein) and Bcl-2 (pro-survival protein). Silencing of Bmi-1 caused a decrease in the Cyclin D1 and Bcl-2, however an increase in p16 was observed. On the contrary, overexpression of Bmi-1 caused an increase in the levels of Cyclin D1, Bcl2- and a decrease in p16 levels. Since Cyclin D1 is a the target of Wnt signaling and Bcl-2 is the major target of Sonic Hedgehog (SHH) signaling, we hypothesize that the Bmi-1 regulates the expression of Cyclin D1 and Bcl-2 by interacting with Wnt /SHH signaling in CaP cells. We have taken steps to understand this mechanism in CaP cells and in this regard further experiments are underway. The successful outcome of these studies will provide deep-insight into the mechanism of CaP cell proliferation and would identify novel molecular targets for CaP chemotherapy.					
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Introduction:

Prostate cancer (CaP) is the most common visceral cancer diagnosed in men; it is the second leading cause of cancer related deaths in males in the United States and the Western world (1). The lack of effective therapies for advanced CaP reflects to a large extent, the paucity of knowledge about the molecular pathways involved in CaP development. Thus, the identification of new predictive biomarkers will be important for improving clinical management, leading to improved survival of patients with CaP. Such molecular targets, especially those that are indicative of proliferation, invasiveness of the disease and survival of cancerous cells (even after chemotherapy) will also be excellent candidate targets for staging the disease and establishing effectiveness of therapeutic and chemopreventive intervention of CaP (2).

The critical pathological processes that occur during the development and progression of human CaP and are known to confer aggressiveness to cancer cells are (i) abolishment of senescence of normal prostate epithelial cells (ii) self-renewability of CaP cells even after chemotherapy and radiation and (iii) dysregulated cell cycle resulting in unchecked proliferation of cancer cells (3-4). Polycomb group (PcG) family of proteins (which form multimeric gene-repressing complexes) have been reported to be involved in self-renewability, cell cycle regulation, and senescence (5-7). Bmi-1 is a transcription repressor originally identified as a c-myc cooperating oncogene in murine lymphoma and has emerged as an important member of PcG family (8). It has been shown to determine the proliferative potential of normal and cancer cells and is reported to be required for the self-renewal of hematopoietic and neural stem cells (9). The human Bmi-1 is located on the short arm of chromosome 10p13, a region known to be involved in translocations in various leukemias and rearrangements in malignant T-cell lymphomas (9). Bmi-1 has been shown to be overexpressed in lymphomas, non-small cell lung cancer, B-cell non-Hodgkin's lymphoma, breast cancer, colorectal cancer and nasopharyngeal carcinoma (10). Bmi-1 has been shown to be a useful molecular marker for predicting occurrence of myelodysplastic syndrome and prognosis of the patients (11). Cellular target genes of Bmi-1 have been identified and include *ink4a* and *ink4b* loci, encoding p16^{INK4A}, p19^{ARF}, and p15^{INK4B} (12). Recently, Glinsky *et al* have shown that the activation of Bmi-1 might be associated with the malignant behavior of CaP cells (13). In the current study, we provide evidence about the over-expression of Bmi-1 in human CaP cells (in particular in highly aggressive and

androgen-independent cell types) and tissue specimens and show that this correlates with the clinical stages of human CaP. We also show that the over expression of Bmi-1 breaks the senescence of normal prostate epithelial cells as well as drives proliferation of CaP cells by regulating the expression of pro-survival and proliferation-associated genes such as Cyclin D1 and Bcl-2. We propose a role for Bmi-1 protein in CaP development and suggest its potential use as a biomarker in the clinical management of CaP.

Body

Under this section we provide information about the experimental design for task # 1 and materials and methods used to accomplish our objectives as stated in the proposal.

Experimental Design for Specific Aim #1.

We conducted the experiments to define the effect of overexpression and silencing of Bmi-1 gene in CaP cells. For this purpose, we (a) knockdown the Bmi-1 gene by transfection of siRNA and (b) overexpressed the Bmi-1 gene by transfecting Bmi-1 construct (pbabe-Bmi-1 plasmid provided by Professor Chi Van Dang, Professor of Cell Biology, School of Medicine, The Johns Hopkins University, Baltimore, MD) in PC3 (androgen-insensitive), LNCaP (androgen-sensitive), CWR22Rv1 (androgen-sensitive) and normal prostate epithelial cells (PrEC) cells. We then studied the growth and viability of transfected cells *in vitro* by employing the MTT assay. To investigate the effect of Bmi-1 gene on the rate of proliferation of CaP cells, we employed $^3\text{[H]}$ thymidine uptake assay. This assay measures the amount of $^3\text{[H]}$ thymidine taken up by dividing cells (for DNA synthesis) thus gives a measure of the rate of division or proliferation of cells. Bmi-1 silenced and Bmi-1 overexpressing CaP cells were cultured in presence of $^3\text{[H]}$ thymidine and $^3\text{[H]}$ thymidine uptake was measured by Liquid scintillation counter. These cells were also measured for DNA content. Further a microarray was performed with Bmi-1 silenced LNCaP cells to understand the mechanism of action of Bmi-1 in CaP cells. Experiments conducted under this aim provided information whether genes involved in proliferation are regulated by Bmi-1 gene. These data were validated by western blot analysis. We analyzed the expression level of Cyclin D1, p16 and Bcl-2 protein in CaP cells. Next we investigated whether the overexpression generates the data contrary to what was observed in Bmi-1 silenced cells. For this purpose Bmi-1 was overexpressed in LNCaP, PC-3 and DU145 cells by transfecting pbabe-Bmi-1 plasmid. Cell lysates prepared from these cells were analyzed for

Cyclin D1, Bcl-2 and p16 proteins by employing western blot analysis. To understand the mechanism through which Bmi-1 regulates Cyclin D1, we carried out experiments on critical pathways which are already known to be associated with Cyclin D1 expression. This includes Wnt/ β -catenin signaling pathway. We asked whether Bmi-1 has any association with Wnt/ β -catenin signaling (which is itself reported to control Cyclin D1). Interestingly, we found that Bmi-1 overexpression causes an increase in the transcriptional activation of *TCF*-responsive element (a bio-marker of Wnt signaling) in CaP cells. Normal prostate epithelial cells are known to replicate between 3-4 cycles normally and after 4 cycles, these enter into a mode of senescence. The break of senescence in normal epithelial cells is a hall mark of progression towards proliferation. This mechanism has high significance in terms of cancer development where normal cells acquire hyper-proliferative characteristics. Since Bmi-1 has been associated with conferring stem-cell like characteristics or self-renewability potential to cancer cells, we next investigated whether Bmi-1 drives the normal prostate epithelial cells (PrEC) towards hyper-replication or proliferation. For this purpose, PrEC cells were transfected with Bmi-1 overexpressing plasmid (pbabe-Bmi-1). As a control to study, another set of PrEC cells were transfected alone vector (pbabe).

Material and Methods:

Cell Lines: Normal prostate epithelial cell (PrEC) was procured from Cambrex. Virally transformed prostate epithelial cells (RWPE-1), PC-3, CWR22Rv1, DU-145 and LNCaP cancer cells were obtained from ATCC (Manassas, VA). Cells were cultured in appropriate media and were kept in a humidified atmosphere of 95% air and 5% CO₂ in an incubator at 37 °C.

Plasmids and siRNA: The pbabe-Bmi-1 plasmid was a kind gift from Dr. Chi V. Dang (The John Hopkins University, Baltimore, MD). pbabe vector was purchased from Addgene Inc. (Cambridge, MA). Bmi-1-siRNA and scrambled siRNA were commercially purchased from Dharmacon (Lafayette, CO).

Immunohistochemistry: Paraffin-embedded sections of human prostate tissues of 125 patients with normal and adenocarcinoma were obtained from the ISU Abxis Co. Ltd., (Seoul, South Korea) and Cybrdi Corporation (Gaithersburg, MD). Immunohistochemical staining was performed using an automated Benchmark immunostainer (Ventana Medical Systems, Tucson, AZ). After antigen retrieval, thick-paraffin-embedded

sections (5 μ m) were dewaxed, rehydrated and endogenous peroxidase activity was blocked. Sections were washed in water and phosphate-buffered saline (PBS) and were blocked in blocking buffer (2% goat serum/5% bovine serum albumin in PBS) for 30 minutes followed by incubation with primary antibody of Bmi-1-specific mAb at the dilution of 1:50 for 1 hour at room temperature. A negative control was included, in which prostate tissues were incubated with normal mouse IgG1 replacing the anti-Bmi-1 monoclonal antibody. After incubation in the primary antibody, sections were washed twice in PBS to remove unbound antibody, followed by incubation for 2 hours at room temperature with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactive complexes were detected using 3,3'-diaminobenzidine (Dako Corp. CA). Slides were then counterstained in haematoxylin, mounted in crystal mount, and cover slipped in 50:50 xylene/Permount. Sections were visualized on a Zeiss-Axiophot DM HT microscope. Images were captured with an attached camera linked to a computer.

Transfections. For siRNA transfection studies, CaP cells were plated at a density of 1×10^6 cells per well in 6-well plates and incubated for 24, 36 and 48 h in complete medium. Using Amaxa nucleofactor kit (Gaithersburg, MD), cells were transfected with siRNAs i.e., non silencing siRNA (100 nM) and Bmi-1 siRNA (100 nM). Cells were harvested after 24, 36 and 48 h and analyzed for expression of Bmi-1. For pbabe-Bmi-1 plasmid transfection studies, CaP cells (1×10^6 cells per well) were transfected with 1-2 μ g of the Bmi-1 construct. For controls, the same amount of empty vector, pbabe and GFP vector (as positive control for transfection) were also transfected. Cells were harvested after 24, 36 and 48 h and analyzed for expression of Bmi-1.

Transcriptional activity of TCF: pTK-TCF-Luc (TopFlash & FopFlash) was procured from Upstate Laboratories (Lake Placid, NY). Cells were transfected with the plasmids (200 ng/well) for 24 h. *Renilla* luciferase (20 ng/well, pRL-TK; Promega, Madison, WI) was used as an internal control. In addition, for controls, the same amount of empty vectors, were transfected in cells. The cells were then harvested and transcriptional activity was measured in terms of luciferase activity in quadruplicates by using dual-luciferase reporter assay system (Promega, Madison, WI).

[³H]-Thymidine incorporation assay: Cells transfected with siRNA's or plasmids were grown in 24 well cluster plates for 24-36 h, the last 16 h of which was in the presence of [³H]thymidine (0.5 µCi/ml). Cells were then washed twice with cold PBS and then were incubated with TCA solution on ice for 30 min and subsequently, the acid-insoluble fraction was dissolved in 1 ml 1M NaOH. The rate of proliferation of CaP cells was calculated by measuring the incorporation or uptake of [³H]thymidine (during cell division) by employing liquid scintillation counter.

Western blot analysis. Cell lysates were prepared in cold lysis buffer [(0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mole/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1 mol/L phenyl methylsulfonyl flouride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The lysate was collected, cleared by centrifugation, supernatant aliquoted and stored at -80 °C. The protein content in the lysates was measured by BCA protein assay (Pierce, Rockford, IL), as per the manufacturers' protocol. For Western blot analysis, 40 µg protein was resolved over 12% Tris-glycine polyacrylamide gels (Novex, Carlsbad, CA) under non-reduced conditions, transferred onto nitrocellulose membranes and subsequently incubated in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20mmol/L TBS, pH 7.6) for 2 hours. The blots were incubated with appropriate primary (human reactive Bmi-1, Cyclin D1, Bcl-2 and p16), washed and incubated with appropriate secondary HRP-conjugated antibody (Amersham Biosciences, Piscataway, NJ). The blots were detected with chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ) and autoradiography, using XAR-5 film (Eastman Kodak, Rochester, NY). Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin (Sigma, St.Louis, MO).

Colony Formation Assay. Cells were transfected with Bmi-1-siRNA and non-silencing siRNA. After 36 h post transfection, cells were harvested, mixed with RPMI containing 10% FCS and 0.5% agar (bottom layer). Cells (5000 cells/well) in 20% FCS and 0.7% Agarose (top layer) were plated and incubated at 37°C for 14 days. After incubation, plates were stained with 0.005% Crystal Violet for more than 2 hours and colonies were counted in two colony grids per well using a microscope.

Focused-microarray. Briefly, 5 µg of RNA was used and cRNA probe was synthesized by labeling the cRNAs with biotin-16-dUTP as per vendor's protocol (Super Array, Frederick, MD). The denatured probes were hybridized with GE array nylon membrane printed with transcripts of 288-well characterized genes and detected by chemiluminescence followed by data analysis with the help of GE array Analyzer.

Statistical analysis. All measures were summarized as means \pm SE. Measures were examined for the appropriateness of a normality assumption by density estimation (data not shown). Associations of categorical variables were evaluated using the Fisher's exact test. Sample correlations were estimated using Spearman's rank correlation. All tests were two-sided and conducted at the $\alpha = 0.05$ significance level. All statistical analyses were performed with the S-plus, Professional Version 6.2 (Insightful Corp., Seattle, WA) software.

Key Research Accomplishments:

We proposed three sub-aims under specific aim # 1 or Task#1. We proposed to accomplish the proposed work in 12 months of the project. Following is the list of work completed as was proposed is Statement of Work:

Proposed Task 1. (A) To establish the involvement of Bmi-1 in the proliferation of human CaP cells (Months 1-15).

Sub Aim-A. *To determine the growth and proliferation of Bmi-1 siRNA-transfected CaP cells.*

Objective (i) : To procure and establish CaP cells in culture (Months 1-2)

Status : CaP cell lines have been procured.

Objective (ii) : To determine the viability, growth and proliferation of cells transfected with Bmi-1 siRNA's by employing MTT assay, trypan blue assay and colony formation assay. (Months 3-4)

Status : We have transfected cells with siRNA's and plasmids. MTT assay, Colony formation and thymidine uptake assay (for measuring rate of proliferation). The data is presented as Figures 2-5 in the annual report.

Objective (iii) : To analyze the effect of Bmi-1-knockdown in CaP cells on the biomarkers of proliferation such as PCNA, BrdU incorporation by employing western blot analysis, immunohistochemical analysis and flow cytometry (Months 4-5).

Status : Cells transfected with scrambled siRNA (control siRNA) and Bmi-1-siRNA were tested for Bmi-1 levels at different time points post transfection by employing western blot analysis. These data are presented as Figure --- in the annual report. Bmi-1-knocked down cells have been tested for evaluating the effect of Bmi-1 suppression on proliferation associated changes by employing micro-array (Table 1). These cells were also tested for proliferation protein levels such as Cyclin D1, Bcl-2 and cell cycle checkpoint protein p16 by employing Western blot analysis. These data are presented as Figure 6 in the annual report. Immunohistochemical analysis of cells and cell cycle analysis by employing flow cytometry experiments are pending because of non-availability of dates to work on Flow Cytometer apparatus. Since several investigators in the university use this centralized facility in the University of Wisconsin, there is a long wait period to get turn to use this machine and to repeat the experiments. However, we are very close to finish these experiments.

Sub-aim- B. *To determine the growth and proliferation of pbabe-Bmi-1 plasmid transfected CaP cells.*

Objective (i) : To transform the pbabe-Bmi-1 plasmid in Top 10 cells and purify it by mini- prep, followed by sequencing and by Maxipreps. (Month 6).

Status : This task has been completed.

Objective (ii): To determine the viability, growth and proliferation of cells transfected with pbabe-Bmi-1 plasmid by employing MTT assay, trypan blue assay and colony formation assay in a dose and time-dependent manner. (Months 7- 8).

Status : We have transfected cells with pbabe-vector and pbabe-Bmi-1 plasmids. We have generated Bmi-1 overexpressing CaP cells. These cells were tested for growth proliferation and clonogenicity by employing MTT assay, Colony formation and thymidine uptake assay (for measuring rate of proliferation). These data are presented as Figures 2-5 in the annual report. In addition, we also investigated the effect of Bmi-1 overexpression on normal prostate epithelial cells.

Objective (iii): To analyze the effect of Bmi-1 overexpression in CaP cells on the biomarkers of proliferation such as PCNA, BrdU incorporation by employing western blot analysis, immunohistochemical analysis and flow cytometry (Months 9-11).

Status : Cells transfected with pbabe vector alone and pbabe-Bmi-1 were tested for Bmi-1 levels by employing western blot analysis. These data are presented as Figure 1 in the annual report. Bmi-1-overexpressing cells were tested for pro-proliferation protein levels such as Cyclin D1, Bcl-2 and cell cycle checkpoint protein p16 by employing Western blot analysis (Figure 6 of the report). Immunohistochemical analysis of cells and cell cycle analysis by employing flow cytometry experiments are pending because of non-availability of dates to work on Flow Cytometer apparatus. Since several investigators in the university use this centralized facility in the University of Wisconsin, there is a long wait period to get turn to use this machine and to repeat the experiments. However, we are very close to finish these experiments.

Sub-aim- C. *To determine the invasive ability of Bmi-1-overexpressed and Bmi-1-silenced CaP cells.*

Objective (i) : To determine whether silencing and overexpressing of Bmi-1 gene in CaP cells change their invasive character in vitro the differences in the invasion rates between control cells and Bmi-1-silenced/or overexpressed cells will be analyzed. (Months 12-13).

Status : This task is in progress. Chemoinvasion chambers required for this experiment were not available on time. However the work is now under progress.

Reportable Outcome

Bmi-1 protein expression in normal and CaP cells: As an attempt towards identifying the expression of Bmi-1 in CaP progression, we first measured protein expression levels by immunoblot analysis in several human prostate carcinoma cell lines, LNCaP, DU-145 and PC-3, and compared them to NHPE and RWPE-1 cells. Among three cell lines used, LNCaP is androgen-sensitive whereas DU-145 and PC-3 are androgen-independent. The choice of these cells was based on the fact that 80% CaP patients present with androgen-dependent disease at the time of diagnosis which later transforms into more aggressive, androgen-independent disease (14-15 and references therein). As shown in Figure 1A, all CaP cell lines exhibited a higher expression

of Bmi-1 protein than in normal prostate epithelial cells. When the protein expression of Bmi-1 was compared among three cancer lines, based on the densitometric analysis of the immunoblots, highly aggressive PC-3 cells and DU145 exhibited 2.5-fold ($p < 0.001$) higher expression than in LNCaP cells. These data suggest a possibility that expression of Bmi-1 protein may be correlated with disease progression and may play a role in aggressiveness of human CaP.

Immunohistochemical analysis of Bmi-1 protein in normal and CaP specimens: In the next series of experiments, we used immunoperoxidase to determine Bmi-1 protein expression in specimens of age-matched normal and CaP representing all tumor stages. In the first experiment, a total of 80 samples were obtained. The staining intensity in tissue specimens were scored on a scale of 0-3. The staining pattern of Bmi-1 protein was compared in grade 1, grade 2 and grade 3 CaP specimens. The mean Bmi-1 expression was 1.5 ± 0.15 (mean \pm S.E; $n = 25$) in grade 1 specimens, 2.5 ± 0.20 (mean \pm S.E; $n = 25$) in grade 2 specimens, and 2.9 ± 0.15 (mean \pm S.E; $n = 30$) in grade 3 specimens (Fig. 5B). These data show a progressive increase of Bmi-1 protein expression corresponding with increasing tumor grade in human CaP (Figure 1B).

Bmi-1 knockdown and Bmi-1 overexpression: Bmi-1 was knocked-down in CaP cells by employing siRNA technique. Cells were analyzed for Bmi-1 protein level at 24 and 36 h post-transfection. Bmi-1 protein levels was observed to be highly reduced at 36 h post-transfection (Figure 2A). This time point was selected for further experiments and biochemical assay utilizing Bmi-1 siRNA transfections. Overexpression of Bmi-1 was achieved in CaP cells by employing transfecting pbabe-Bmi-1 plasmid in CaP cells. Cells were analyzed for Bmi-1 protein level at 24 and 36 h post-transfection. Bmi-1 protein level was observed to be highly increased at 36 h post-transfection in CaP cells. The data representing Bmi-1 protein at 36h is presented in Figure 2B. This time point was selected for further experiments and biochemical assay utilizing Bmi-1 siRNA transfections.

Effect of Bmi-1 knockdown and Bmi-1-overexpression on the growth of CaP cells: Next we investigated effect of Bmi-1 on the growth and viability of CaP cells by employing MTT assay. LNCaP cells are known to duplicate under culture conditions from 48-72 h. Similarly DU145 and PC-3 cells duplication takes 24 h under culture conditions. Culture dishes containing LNCaP, DU145 and PC-3 cells become confluent between 48-72 h. It is noteworthy that Bmi-1-silenced CaP cells did only grow between 50-65% even after 72 h post-

transfection (Figure 3A). On the contrary, Bmi-1-overexpressing CaP cells exhibited significantly increased growth (Figure 3B). At 36 h post transfection control LNCaP cells displayed 35% growth while as Bmi-1 overexpressing LNCaP cells displayed 60 % growth. Similarly Bmi-1-overexpressing DU145 and PC-3 cells exhibited 75-100 % cell confluency at 36 h post-transfection as compared to control which exhibited 50% confluency (Figure 3B). These data suggest the importance of Bmi-1 in the growth of CaP cells.

Effect of Bmi-1 knockdown and Bmi-1-overexpression on proliferation of CaP cells. We investigated whether Bmi-1 regulates the proliferation process of CaP cells. We employed a two-way approach where Bmi-1 was either knocked down or overexpressed in CaP cells and such CaP cells were later assessed for their proliferative and clonogenic potential by employing ³[H]thymidine uptake assay. Firstly, LNCaP, PC-3 and DU145 cells were transfected with Bmi1-siRNA (100 nM). To investigate the effect of Bmi-1 gene suppression on the rate of proliferation of LNCaP, DU145 and PC-3 cells, we performed ³[H]thymidine uptake assay. Suppression of Bmi1-1 expression resulted in the decreased rate of proliferation of CaP cells (Figure 4A). Bmi-1-overexpressing CaP cells displayed significantly increased rate of proliferation (Figure 4B).

Colony formation assay is used as a marker for proliferation of cells under *ex-vivo* conditions. Next, we investigated the effect of suppression of Bmi-1 on the clonogenic potential of CaP cells in *ex-vivo* conditions. For this purpose we employed a soft agar colony assay and assessed the clonogenic potential of CaP cells transfected with Bmi-1 siRNA and pbabe-Bmi-1 plasmid. Bmi-1 knockdown resulted in a significant reduction in the number of colonies formed by CaP cells as compared to control siRNA-treated cells (Figure 5A). However, Bmi-1-overexpressing CaP cells exhibited increased clonogenicity potential as is evident from an increase in the average number of colonies formed by Bmi-1-overexpressing cells (Figure 5B). These data suggested that Bmi-1 confers proliferative attributes to the CaP cells.

Effect of Bmi-1 gene knockdown on CaP-associated genes: Next, we investigated the mechanism through which Bmi-1 controls the proliferation and survival of CaP cells. We performed a focused microarray analysis of 288 well-characterized proliferation and survival-associated genes in Bmi-1 suppressed-CaP cells. The list of genes that were observed to exhibit changes in their expression pattern in response to Bmi-1 knockdown in CaP cells is presented in Table 1. Most notably, we observed that suppression of Bmi-1 gene in CaP cells caused a

significant reduction (> 95%) in the expression of Cyclin D1, Bcl-2, urokinase plasminogen activator (uPA), matrix metalloproteinase (MMP)-9 and nuclear factor kappa B (NFκB) (Table 1). We also observed an increased expression of p16, p15 and TIMP-3 (Table 1). Since Cyclin D1 and Bcl-2 were highly responsive to Bmi-1 gene suppression; we selected these genes for further biochemical studies.

Effect of Bmi-1 gene knockdown and Bmi-1-overexpression on Cyclin-D1 and Bcl-2 Levels: Increased Cyclin-D1 activity and Bcl-2 are considered important for the increased proliferation and survival of cancerous cells (16-21). Next, we analyzed the effect of Bmi-1 gene-suppression on the expression of cyclin D1 and Bcl-2 in LNCaP, DU145 and PC-3 cells. Bmi-1 knockdown caused a decrease in the expression level of Cyclin D1 and Bcl-2 protein in CaP cells (Figure 6A). Next we determined the effect of Bmi-1 overexpression on the expression levels of cyclin D1 and Bcl-2. CaP cells transfected with Bmi-1 construct exhibited a significant increase in the expression level of cyclin D1 and Bcl-2 protein (Figure 6B). These data were consistent with microarray data suggesting a possible association between the Bmi-1, Cyclin D1 and Bcl-2 during progression of human CaP.

Effect of Bmi-1-overexpression on transcriptional activation of TCF-responsive element: Cyclin D1 expression has been reported to be regulated by Wnt signaling (22). Since we observed that Bmi-1 also regulate Cyclin D1 expression, we asked whether there is any association between Bmi-1 and Wnt signaling. Next we investigated effect of Bmi-1 overexpression on Wnt signaling by evaluating the transcriptional activation of *Tcf-responsive* element (marker of Wnt signaling) by employing luciferase reporter assay. It is noteworthy that Bmi-1 over-expression caused an increase in the transcriptional activation of TCF-responsive element suggesting that Bmi-1-induced Cyclin D1 expression might be through the activation of Wnt signaling (Figure 7). This is the first report where Bmi-1 is shown to regulate Wnt signaling. To fully understand the association between Bmi-1 and Wnt signaling, the work is underway.

Effect of Bmi-1 overexpression on the replicative life of normal prostate epithelial cells (PrEC): Transfection of pbabe-Bmi-1 plasmid significantly increased the replicative life of PrEC cells upto 8 passages which

generally enters into a stage of senescence after 4-5 divisions (Figure 8). These data suggest that Bmi-1 possess the potential to drive normal cells towards proliferation.

Conclusion

Recent experimental observations documented an increased Bmi-1 expression in human non-small-cell lung cancer, human breast carcinomas, and established breast cancer cell lines, suggesting that an oncogenic role of Bmi-1 activation may be extended beyond leukemia and, perhaps, may affect progression of the epithelial malignancies as well (8-13, 23-24). Over expression of Bmi-1 is reported to confer invasive potential to glioma and breast cancer cells, and cause malignant phenotype in rat and human cancer cells, however, the role of Bmi-1 in human CaP metastasis is yet to be elucidated (8-13, 23-24). In the present study, we observed that Bmi-1 gene controls the invasiveness and growth of human CaP cells under *in vitro* and *in vivo* conditions through the regulation of Cyclin D1 and Bcl-2. To our knowledge, this report is the first demonstration that Bmi-1 regulates Cyclin D1 and Bcl-2 in human CaP cells.

In our preliminary studies (data generated in the first year of the project), we show that Bmi-1 protein levels are elevated in CaP patients if high grade tumor. Further, we also show that overexpression of Bmi-1 gene increases the rate of proliferation and invasion of CaP cells and its suppression reverses this effect. We provide evidence that suppression of Bmi-1 gene reduces the proliferative and clonogenic potential of human CaP cells. On the contrary, overexpression of Bmi-1 was observed to increase the clonogenic potential of human CaP cells.

Cyclin D1 is a critical protein that is required for cell division and proliferation. Cyclin D1 levels have been reported to be increased during several cancer types (25-33). CaP patients have been shown to exhibit increased Cyclin D1 levels (34-35), however the mechanism through which Cyclin D1 levels are altered during cancer development is not fully understood. In this study, we provide evidence that Cyclin D1 is regulated by Bmi-1 in CaP cells. It is noteworthy that CaP cells deficient in Bmi-1 exhibited decreased Cyclin D1 levels. However, Bmi-1-overexpressing CaP cells displayed an increase in Cyclin D1 protein levels suggesting significance of Bmi-1 protein for Cyclin D1 expression in CaP cells.

To understand the mechanism through which Bmi-1 regulates Cyclin D1, we studied critical pathways which are known to be associated with Cyclin D1 expression. This includes Wnt/ β -catenin signaling pathway. We asked whether Bmi-1 has any association with Wnt/ β -catenin signaling (which is itself reported to control Cyclin D1). Interestingly, we found that Bmi-1 overexpression causes an increase in the transcriptional activation of TCF-responsive element (a bio-marker of Wnt signaling) in CaP cells. These data are highly significant. This finding is novel and is the first report showing that Bmi-1 regulates Wnt signaling in CaP cells. Wnt signaling is report to be involved in the proliferation and chemoresistance of CaP cells. Further work to understand the mechanistic role in Wnt signaling in CaP cells is underway and will be completed by the end of 2nd year of the proposed project.

Bcl-2 protein is known to play an important role in the survival of cancer cells by conferring anti-apoptotic potential to cells (18-21). Bcl-2 levels are reported to be high in cancer cells including CaP cells (36). In the current study, we provide evidence that Bmi-1 is associated with expression of Bcl-2 in CaP cells. Bmi-1-deficient CaP cells exhibited decreased Bcl-2 levels while as Bmi-1-overexpressing CaP cells exhibited increased Bcl-2 levels. As evident from reports which suggest that Bmi-1 confers renewability or stemness characteristics to cancer cells and Bcl-2 confers survivability characteristics to cancer cells, our data showing association between Bmi-1 and Bcl-2 is highly significant. However, it would be important to fully understand the mechanism through which Bmi-1 regulates Bcl-2 in CaP cells. To understand the mechanism through which Bmi-1 regulates Bcl-2 in CaP cells, we have planned experiments and some of experiments are in progress. These will be completed by the end of 2nd year of the proposed project.

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Legends to Figures


Figure 1. Bmi-1 protein levels in (A) normal prostate epithelial cells, CaP cells and (B) human prostatic tumor tissues. (A) Expression of Bmi-1 protein in NHPE, RWPE1 and prostate cancer cells LNCaP, DU145 and PC-3 by western blotting. Equal loading of protein was confirmed by stripping the blots and reprobing with β -actin antibody. The histogram indicates the relative density of the bands normalized to β -actin. Representative data for five experiments are shown here. Each bar represents mean of relative densities \pm S.E. NS, non-significant. (B) Immunostaining for matriptase in representative specimens of CaP specimens of tumor stages I-III and non-neoplastic regions of prostatic specimens of CaP patients. CaP specimens were assigned tumor grades on the basis of Gleason pattern and Gleason score as described in Materials and Methods. Immunoreactive Bmi-1 protein was observed in a coarsely granular pattern in cell cytoplasm of epithelial cells of grade 1, grade 2 and grade 3 prostatic adenocarcinoma. There was minimal staining of occasional stromal cells in non-neoplastic regions. Bmi-1 expression was weak in normal and moderate to strong in advanced CaP specimens.  Arrows indicate staining for Bmi-1 in cancer regions. Magnification X 40.

Figure. 2. Effect of Bmi-1-siRNA and pbabe-Bmi-1 plasmid transfection on the expression levels of Bmi-1 protein in CaP cells: (A) Immunoblots represent the effect of Bmi-1-siRNA transfection on the expression level of Bmi-1 protein in LNCaP, DU145 and PC-3 cells. Cells were transfected with 100 nM of siRNA directed against Bmi-1 and scrambled siRNA (100 nM). Cells were harvested at 24 and 36 h post transfection. Cell lysates were prepared as described under Materials and methods. The expression level of Bmi-1 was determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β -actin. (B) Immunoblots represent the effect of pbabe-Bmi-1 plasmid transfection on the expression level of Bmi-1 protein in LNCaP, DU145 and PC-3 cells at 36 h post transfection. Cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). Cells were harvested at 24 and 36 h post transfection. Cell lysates were prepared as described under Materials and methods. The expression level of Bmi-1 was determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β -actin.

Figure 3. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the growth and viability of CaP cells:

(A) The histogram represents the % viability of human CaP cells LNCaP, DU145 and PC-3 at 72 h post-transfection as measured by MTT assay. (B) The histogram represents the % viability of human CaP cells LNCaP, DU145 and PC-3 at 36 h post-transfection as measured by MTT assay.

Figure 4. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the rate of proliferation of CaP cells:

(A) Histogram showing rate of ^3H thymidine uptake in Bmi-1-silenced CaP cells, LNCaP, DU145 and PC-3. Cells were transfected with Bmi-1-siRNA (100nM). Control cells were transfected with scrambled siRNA (100nM). Cells were incubated for 36h, the last 16 h of which were in the presence of ^3H thymidine (0.5 $\mu\text{Ci/ml}$). Each bar represents mean \pm SE of three independent experiments. *indicates $p < 0.05$. (B) Histogram showing rate of ^3H thymidine uptake in Bmi-1-overexpressed CaP cells, LNCaP, DU145 and PC-3. Cells were transfected with pbabe-Bmi-1 (2 μg). Control cells were transfected with pbabe vector alone (2 μg). Cells were incubated for 36h, the last 16 h of which were in the presence of ^3H thymidine (0.5 $\mu\text{Ci/ml}$). Each bar represents mean \pm SE of three independent experiments. *indicates $p < 0.05$.

Figure 5. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the clonogenic potential of CaP cells.

(A) Histogram showing number of colonies formed by Bmi-1-silenced LNCaP, DU145 and PC-3 cells. Bmi-1-silenced cells were seeded in agarose and incubated at 37°C as described under Materials and methods. After 10 days of incubation, the cells were stained with crystal violet/methanol and colonies were counted. Each bar in the histogram represents mean \pm S.E., * indicates $p < 0.05$. All experiments were repeated three times with similar results. (B) Histogram showing number of colonies formed by Bmi-1-overexpressing LNCaP, DU145 and PC-3 cells. Bmi-1-overexpressing CaP cells were seeded in agarose and incubated at 37°C as described under Materials and methods. After 10 days of incubation, the cells were stained with crystal violet/methanol and colonies were counted. Each bar in the histogram represents mean \pm S.E., * indicates $p < 0.05$. All experiments were repeated three times with similar results.

Figure 6. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the expression level of prominent proliferation-associated proteins in CaP cells. (A) Immunoblots represent the effect of Bmi-1-knockdown on

the expression level of Cyclin D1, Bcl-2 and p16 proteins in LNCaP, DU145 and PC-3 cells. Cells were transfected with 100 nM of siRNA directed against Bmi-1 and scrambled siRNA (100 nM). Cells were harvested at 36 h post transfection. Cell lysates were prepared as described under Materials and methods. The expression level of Cyclin D1, Bcl-2 and p16 were determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β -actin. **(B)** Immunoblots represent the effect of Bmi-1-overexpression on the expression level of Cyclin D1, Bcl-2 and p16 proteins in LNCaP, DU145 and PC-3 cells. Cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). The expression levels of proteins were determined by western blot analysis. Equal loading was confirmed by probing the immunoblots for β -actin. The immunoblots shown here are representative of three independent experiments with similar results.

Figure 7. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the transcriptional activation TCF-responsive element in CaP cells. Histogram represents the effect of Bmi-1 over-expression on the transcriptional activation of TCF responsive element (marker of Wnt/ β -catenin signaling) in LNCaP, DU145 and PC-3 cells. CaP cells were transfected with pTK-TCF-Luc (pTopFlash)-constructs. pFopFlash and *Renilla* luciferase were used as negative and internal control respectively. For controls, the same amount of empty vectors, were transfected in cells. The transcriptional activity was measured in terms of luciferase activity as described under Materials and methods. Relative luciferase activity was calculated with the values from vector alone group.

Figure 8. Effect of Bmi-1 over-expression on the rate of replication or proliferation of normal prostate epithelial cells (PrEC): Micrographs showing the morphology of Bmi-1 overexpressing PrEc cells. PrEC cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). Confluent dishes containing Bmi-1-overexpressing cells and vector-transfected PrEC cells were split or seeded after every 36 h. (upper Panel) Cell splitting or seeding continued for 4 passages or replication cycles in pbabe-transfected PrEC cells and did not duplicate after 4 passages and entered into senescence phase.(Lower Panel) Cell splitting or seeding continued for 8 passages or replication cycles in pbabe-transfected PrEC cells and cell replication

continued upto 4 passages. Since this was a transient transfection and the overexpression effect of Bmi-1 lasted upto 8th passage only. Cells entered into senescence phase at 9th passage. Inset regions (400X) showing cells with senescent morphology features of live cells such as globular shape.

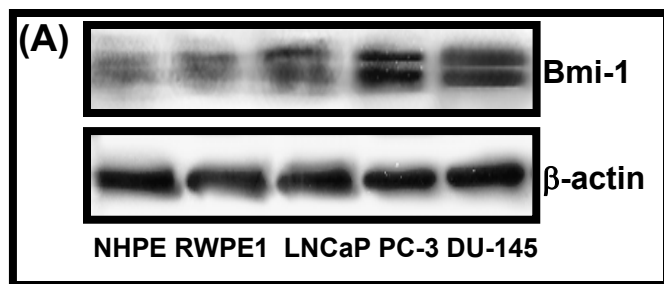
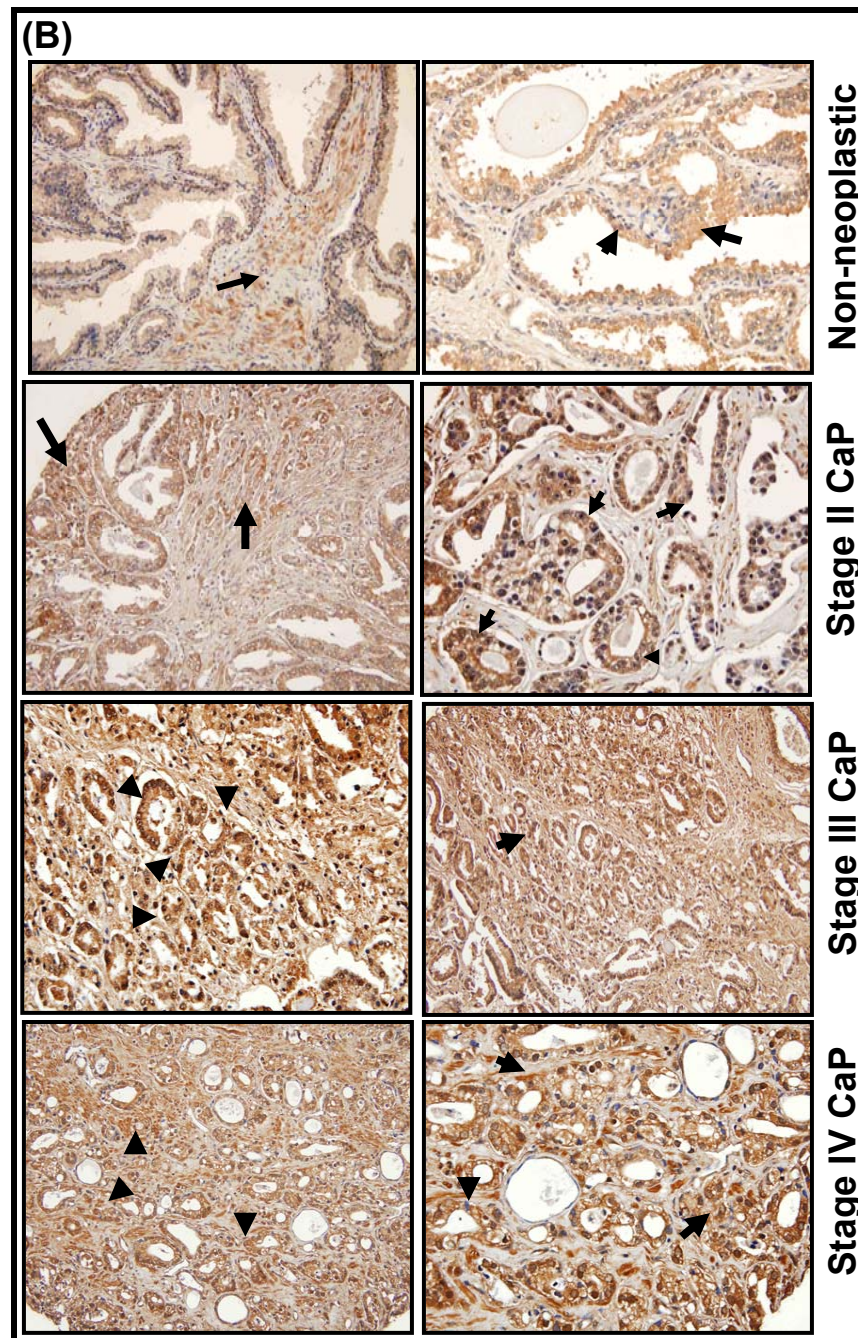


Figure 1



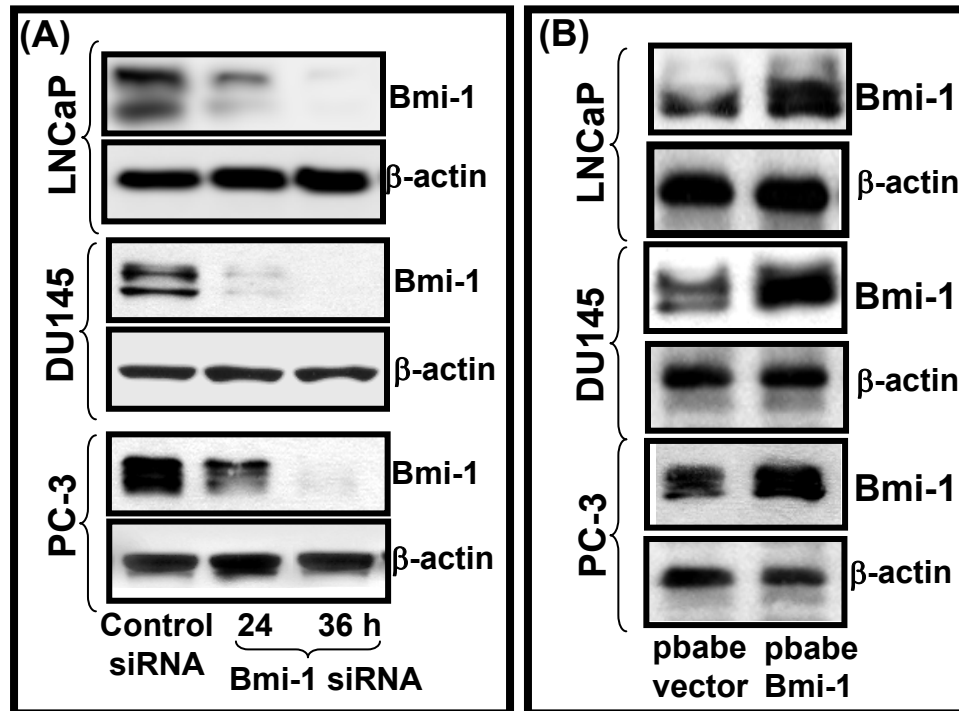


Figure 2

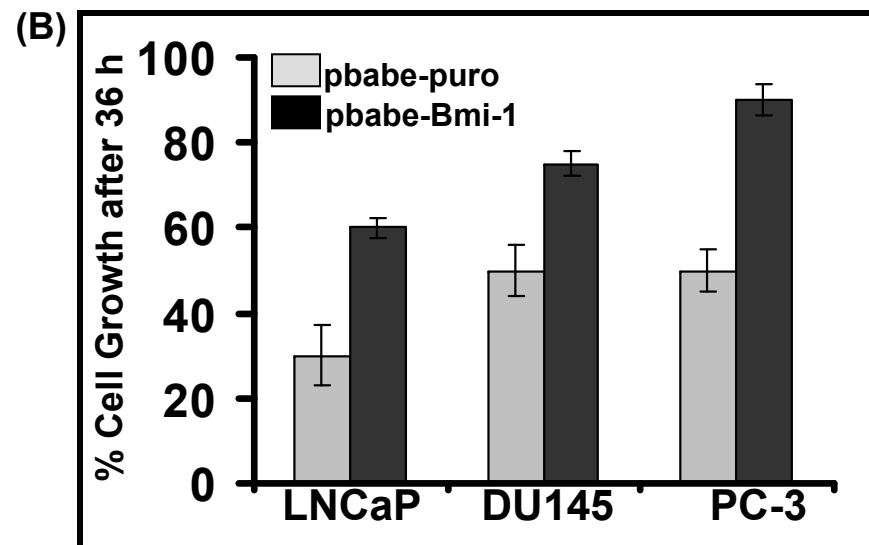
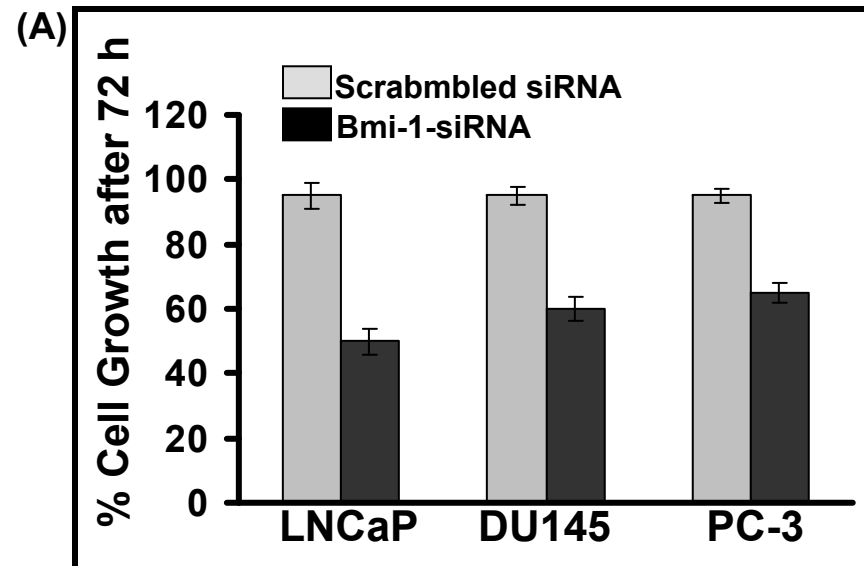


Figure 3

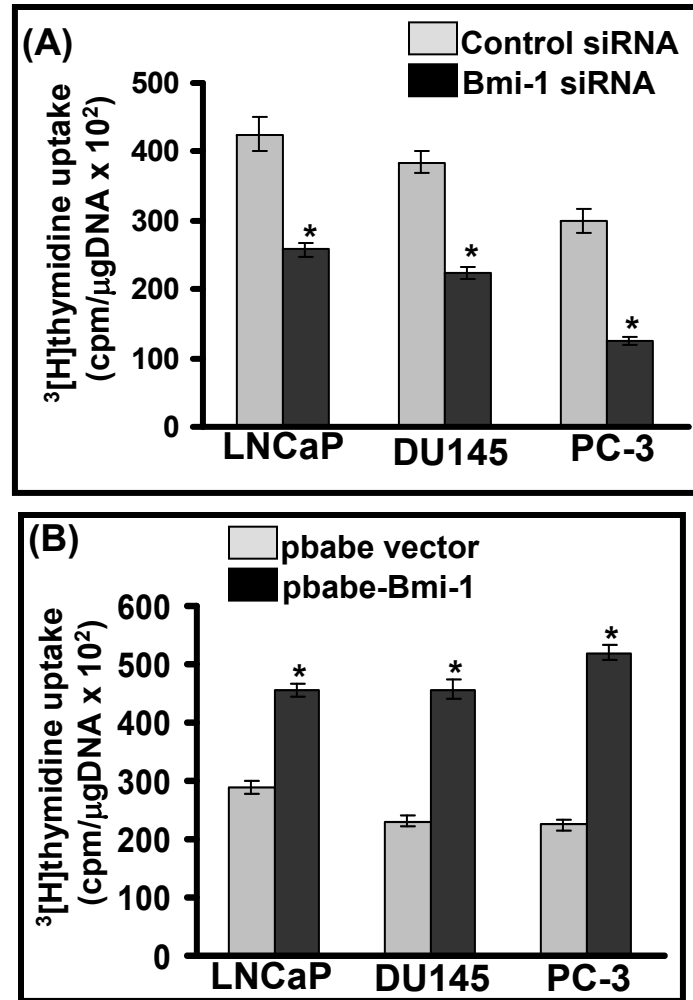


Figure 4

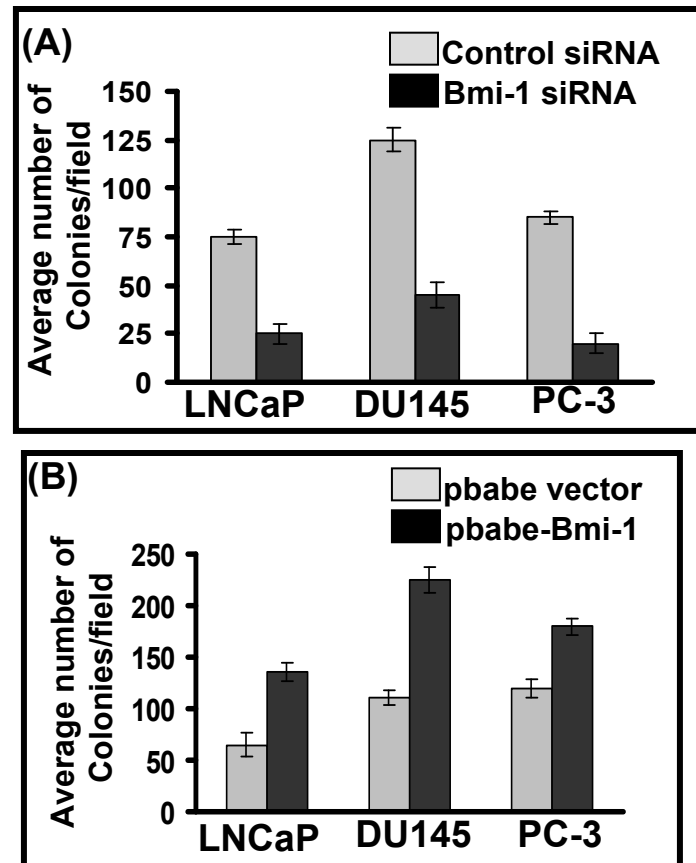


Figure 5

Table 1. Effect of Bmi-1 knockdown on Expression of Proliferation-Associated Genes in CaP Cells

Gene Bank Accession Number	Gene Description	Gene status after Bmi-1-siRNA transfection
NM_053-56	Cyclin D1	Down-regulated **
NM_000075	Cyclin-dependent kinase 4 (Cdk4)	Down-regulated **
NM_000657	Bcl-2	Down-regulated **
NM_005163	Akt1	Down-regulated *
NM_004570	PI3K (catalytic gamma peptide)	Down-regulated *
NM_002658	Urokinase plasminogen activator	Down-regulated **
NM_001005862	ErbB2	Down-regulated **
NM_005417	Src	Down-regulated *
NM_002467	c-myc	Down-regulated **
NM_002228	c-Jun	Down-regulated *
NM_005343	H-Ras	Down-regulated *
NM_001033756	Vascular endothelial growth factor (VEGF)	Down-regulated **
NM_0004994	Matrix metalloproteinase -9 (MMP-9)	Down-regulated **
NM_003998	Nuclear factor kappa B 1(NFκB1)	Down-regulated *
NM_001530	HIF-1	Down-regulated *
NM_000875	Insulin growth factor 1(IGF1)	Down-regulated **
NM_0008756	Insulin growth factor 1(IGF2)	Down-regulated **
NM_000586	Interlukin 2(IL-2)	Down-regulated **
NM_013430	GGT2	Down-regulated *
NM_006254	Protein kinase C delta (PKC δ)	Down-regulated *
NM_005400	Protein kinase C epsilon (PKC ε)	Down-regulated*
NM_058195	p16/INK4	Up-regulated **
NM_004936	p15	Up-regulated **
NM_000076	p57	Up-regulated **
NM_006016	CD164	Up-regulated *
NM_016279	Cadherin-9	Up-regulated *
NM_000362	TIMP-3	Up-regulated *
NM_173206	PIAS2	Up-regulated **

* Represents 2- 5 fold and ** represents more than 5 fold.

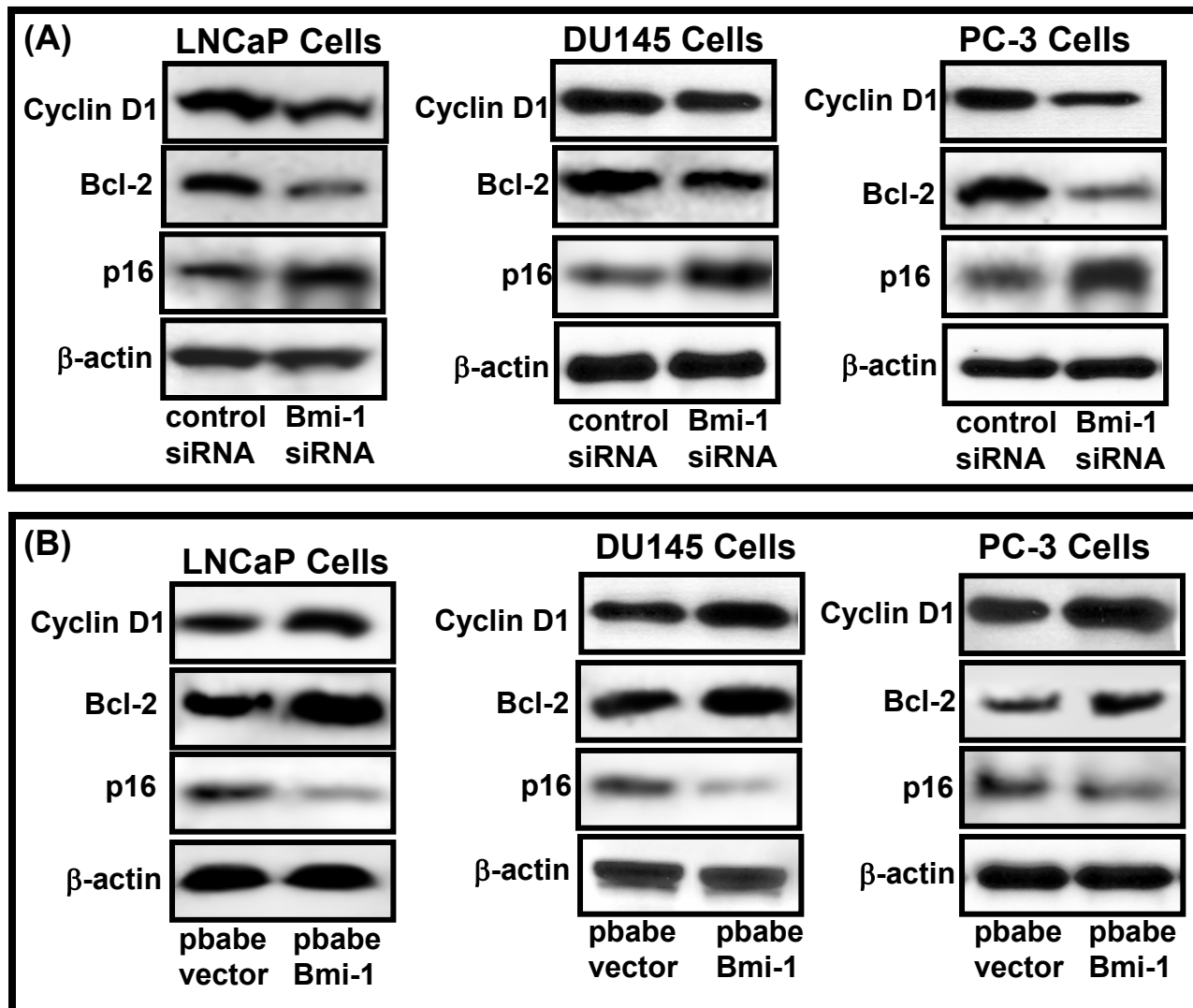


Figure 6

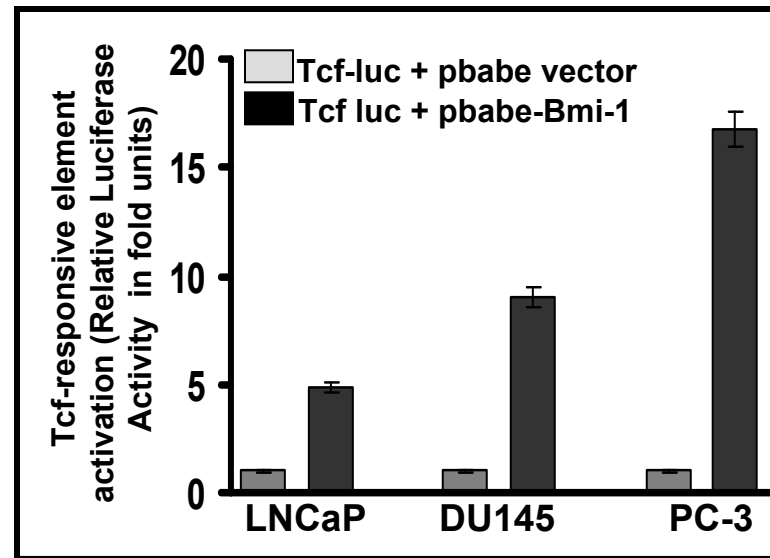


Figure 7

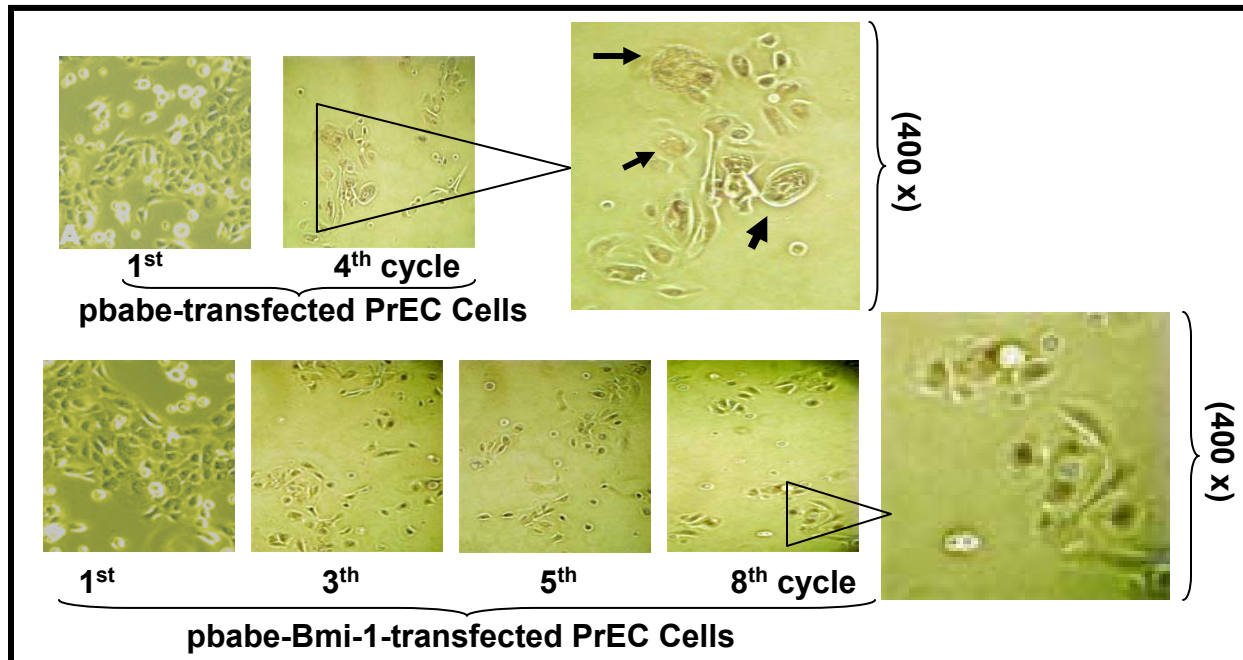


Figure 8